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(54) Title: PROCESSING OF PEPTIDES AND PROTEINS

(57) Abstract: The invention provides novel methionine aminopeptidase enzymes and their use.

PROCESSING OF PEPTIDES AND PROTEINS

FIELD OF THE INVENTION

The invention relates to a method for processing initiator methionine containing proteins by the enzyme Methionine Aminopeptidase and mutants thereof to yield initiator methionine free peptides.

BACKGROUND OF THE INVENTION

Production of peptides by recombinant techniques using either prokaryotic or eukaryotic expression systems inherently yields the peptide with a leading methionine amino acid. This amino acid may not be present in the native protein i.e. the variant of the peptide processed for translocation. Obtaining the peptide without the leading methionine thus requires a further processing step. In the present invention the step is performed by the enzyme Methionine Aminopeptidase, which selectively cleaves the initiator methionine from the peptide.

Methionine Aminopeptidases (Met-AP's) are known in the art as enzymes which cleaves leading methionines, if the leading peptide sequence is of a certain predetermined character. Wild-type *Escherichia coli* Met-AP selectively cleaves after an initiator Met residue if the residue in the P1' position is Gly, Ala, Ser, Thr, Pro, Val or Cys.

In the present invention the methionine aminopeptidases are improved by introducing mutations in the substrate binding sites which results in methionine aminopeptidases which cleaves the methionine regardless of the leading peptide sequence (P1' position)

SUMMARY OF THE INVENTION

The invention provides novel mutant methionine aminopeptidases.

The invention provides isolated DNA encoding such methionine aminopeptidases.

The invention provides host cells for producing such methionine aminopeptidases.

The invention provides the use of the mutant methionine aminopeptidase for processing of peptides with an initiator methionine amino acid into a methionine free peptide.

The invention also provides the processing of specific peptides by mutant methionine aminopeptidases.

The invention also provides a method for separating the methionine containing starting material from the final cleaved product.

DESCRIPTION OF THE DRAWINGS

Figure 1: Example of a *E. coli* Met-AP mutant expression construct layout. Purification indicates tag for purification purposes. Protease indicates protease cleavage site.

Figure 2: Expression in e.g. *E. coli* of (A) NT1-Enterokinase-Met-AP Y168A or (B) NT1-Enterokinase-Met-AP Y168G, M206N, Q233A as indicated.

Figure 3: hexa-His-Xa-Met-AP (M206A, Q233A) cleavage of Met-hIL-21

Figure 4: Maldi-tof es mass spectrum of purified hexa-His-Xa-Met-AP M206A, Q233A.

Figure 5: Purification chromotogram of the separation of the three compounds Met-IL-21, IL-21 and pyroglutamine IL-21.

DEFINITIONS

P1 defines the first amino acid N-terminal to the recognition site for the enzyme. P1' denotes the amino acid adjacent to P1 towards the C-terminal. P1 in the present invention is methionine.

In the present invention substrate specificity means selectivity towards the P1' position – which is the position just C-terminal to methionine. Wild-type *Escherichia coli* Met-AP exhibits the substrate specificity, that it selectively cleaves after an initiator Met residue if the residue in the P1' position is Gly, Ala,

Ser, Thr, Pro, Val or Cys. The mutants of the present invention showing an extended substrate specificity means that further aminoacid can occupy the P1' position and still cleavage of the methionine is observed.

In the context of the present invention variant means a sequence which has maintained the qualitative activity of the parent sequence, ie as methionine aminopeptidase, but wherein the sequence differs from the parent sequence by deletions, insertions, extension or substitution of one or more amino acids of the parent sequence. Variants in principle also includes fragments of any length provided the activity is maintained.

In the context of the present invention chemical derivatives of a specific protein means a derivative of the native protein which is not a variant, and which maintains the qualitative activity of the parent protein sequence. The chemical derivative includes derivatives such as PEG-groups.

The terms peptide and proteins are used interchangeable and is not meant as indications or limitations as to size or function of the sequences.

DESCRIPTION OF THE INVENTION

The Met-Ap from *E. coli* has a substrate defining pocket (as part of active site) essentially, but probably not exclusively, defined by the amino acids Tyr 168, Met 206 and Gln 233. Mutating these positions extends the enzymes substrate specificity. The novel E.coli aminopeptidases described in the present invention extends the applicability of the Methionine aminopeptidases to be useful for removing the initiating methionine from almost any type of protein or peptide regardless of the amino acid sequence downstream of the methionine (P1' position). Hence, the initiator methionine can be removed from all initiator methionine containing peptides or proteins to produce initiator methionine-free peptides or proteins.

The *E. coli* Methionine aminopeptidase gene was cloned and mutant versions have been created using site directed mutagenesis.

The mutants were expressed in *E. coli* and the resulting enzymes were purified by conventional His-tag system. The enzyme can also be tagged by for example the FLAG-system or tagged and purified by other technologies as de-

scribed in WO 03042249. Catalytic activity was monitored using initiator Met containing hIL-21 as a substrate.

In principle, the invention is generally applicable to any peptide. The invention is demonstrated as being useful for cleavage of the initiator methionine for peptides such as hIL-21. hIL-21 is a model system for P1' position being a Gln. IL-21 is described in WO00/53761 and is described as being effective in the treatment of cancer and viral infection among others. IL-20 is described in WO9927103. hGH refers to human Growth Hormone. Both are model systems for other aminoacids in P1' position.

In an aspect the invention provides *E. coli* aminopeptidease variants which are mutated in the active site having extended substrate specificity the P1' position relative to the wild type.

In an aspect the invention provides the E.coli methionine aminopeptidase variants as described above which extend the substrate specificity in P1' position to include Asn, Leu, Ile, Phe, His, Gln or Trp as well as the aminoacids allowed in position P1' by the wild type.

In an aspect the invention provides E.coli methionine aminopeptidases as described above, wherein the residues in positions 168, 206 or 233 has been amended into a sequence different from Y168 and/or M206 and/or Q233.

In an aspect the invention provides E.coli methionine aminopeptidases as described above, comprising amendments of the amino acid in position 168.

In an aspect the invention provides E.coli methionine aminopeptidases as described above comprising amendment in position 206.

In an aspect the invention provides E.coli methionine aminopeptidases as described above comprising amendment in position 233.

In an aspect the invention provides E.coli methionine aminopeptidases as described above, comprising amendments in position 206 and 233.

In an aspect the invention provides E.coli methionine aminopeptidases as described above comprising amendments in position 168 and 206.

In an aspect the invention provides E.coli methionine aminopeptidases as described above comprising amendments in position 168 and 233.

In an aspect the invention provides E.coli methionine aminopeptidase as described above, comprising amendments in positions 168, 206 and 233.

In an aspect the invention provides E.coli methionine aminopeptidases as described above, wherein the amendments comprises exchange of wildtype amino acid into Gly, Ala, Ser, Thr, Asn or Asp.

In an aspect the invention provides E.coli methionine aminopeptidases as described above, wherein the amendments comprises Ala and/or Gly.

In an aspect the invention provides E.coli methionine aminopeptidases as described above, wherein the amendments comprises Ala.

In an aspect the invention provides E.coli methionine aminopeptidase as described above, wherein position 168 is Ala.

In an aspect the invention provides E.coli methionine aminopeptidase as described above, wherein position 206 is Ala.

In an aspect the invention provides. E.coli methionine aminopeptidase as described above; wherein position 233 is Ala.

The invention thus provides the methionine aminopeptidase enzyme having the following sequence (also described as seq. id. no. 1)

MAISIKTPEDIEKMRVAGRLAAEVLEMIEPYVKPGVSTGELDRICNDYIVNEQHAVSACLGY
HGYPKSVCISINEVVCHGIPDDAKLLKDGDIVNIDVTVIKDGFHGDTSKMFIVGKPTIMGER
LCRITQESLYLALRMVKPGINLREIGAAIQKFVEAEGFSVVRE**Xa** CGHGIGRGFHEEPQVLH
YDSRETNVVLKPGMTFTIEP**Xb** VNAGKKEIRTMKDGWTVKTKDRSLSA**Xc** YEHTIVVT
DNGCEILTLRKDDTIPAIISHDE,

wherein X_a, X_b and X_c are variable amino acids, and wherein X_a, X_b and X_c are not simultaneously Tyr, Met and Gln respectively. In an aspect of the invention one or more of X_a, X_b and X_c are exchanged from the wild type amino acid into Gly, Ala, Ser, Thr, Asn or Asp. In an aspect of the invention X_a, X_b and X_c are exchanged from the wild type amino acid into Gly or Ala; . In an aspect of the invention X_a, X_b and X_c are exchanged from the wild type amino acid into Ala. The present invention thus provides substitution Y168 to Ala (Y168A)(Seq. id no. 9)

MAISIKTPEDIEKMRVAGRLAAEVLEMIEPYVKPGVSTGELDRICNDYIVNEQHAVSACL-
GYHGYPKSVCISINEVVCHGIPDDAKLLKDGDIVNIDVTVIKDGFHGDTSKMFIV-
GKPTIMGERLCRITQESLYLALRMVKPGINLREIGAAIQKFVEAEGFSVVREACGHGIGRG-
FHEEPQVLHYDSRETNVVLKPGMTFTIEPMVNAGKKEIRTMKDGWTVKTKDRSLSA-
QYEHTIVVTDNGCEILTLRKDDTIPAIISHDE

and the corresponding DNA encoding the above as seq. id. no. 8;

The present invention thus provides substitution Met 206 to Ala (M206A)(Seq. id no. 3)

MAISIKTPEDIEKMRVAGRLAAEVLEMIEPYVKPGVSTGELDRICNDYIVNEQHAV
SACLGYHGYPKSVCISINEVVCHGIPDDAKLLKDGDIVNIDVTVIKDGFHGDTSKMFIVGKP
TIMGERLCRITQESLYLALRMVKPGINLREIGAAIQKFVEAEGFSVVREXCGHGIGRGFHEE
PQVLHYDSRETNVVLKPGMTFTIEPAVNAGKKEIRTMKDGWTVKTKDRSLSAQYEHTIVVT
DNGCEILTLRKDDTIPAIISHDE,

which extends the enzymes substrate specificity to allow the following amino acids: Asn, Leu, Ile and Phe in the P1' postion.

The present invention also provides substituting Gln 233 to Ala (Q233A) (Seq. id. No.5)

MAISIKTPEDIEKMRVAGRLAAEVLEMIEPYVKPGVSTGELDRICNDYIVNEQHAV
SACLGYHGYPKSVCISINEVVCHGIPDDAKLLKDGDIVNIDVTVIKDGFHGDTSKMFIVGKP
TIMGERLCRITQESLYLALRMVKPGINLREIGAAIQKFVEAEGFSVVREXCGHGIGRGFHEE
PQVLHYDSRETNVVLKPGMTFTIEPMVNAGKKEIRTMKDGWTVKTKDRSLSAAYEHTIVVT
DNGCEILTLRKDDTIPAIISHDE,

or both Met 206 and Gln 233 into Ala (M206A Q233A)(Seq. id no. 7):

MAISIKTPEDIEKMRVAGRLAAEVLEMIEPYVKPGVSTGELDRICNDYIVNEQHAV-
SACLGYHGYPKSVCISINEVVCHGIPDDAKLLKDGDIVNIDVTVIKDGFHGDTSKMFIV-
GKPTIMGERLCRITQESLYLALRMVKPGINLREIGAAIQKFVEAEGFSVVREXCGHGIGRG-
FHEEPQVLHYDSRETNVVLKPGMTFTIEPAVNAGKKEIRTMKDGWTVKTKDRSLSAAYEHT
IVVTDNGCEILTLRKDDTIPAIISHDE,

which further allow the P1' amino acids to be His, Gln and Trp.

In aspects of the invention postion 168 is amended into Gly (Y168G) or Ala (Y168A) or Asn (Y168N). Aspects of the invention are wherein amino acid 206 is an Ala (M206A) or a Gly (M206G) or Asn (M206N), and/or wherein amino acid 233 is an Ala (Q233A) or a Gly (Q233G) or Asn (Q233N). Aspects of the invention comprise the combination of two or three amendments according to the below, - wherein the wild-type combination of (Y168 M206 233Q) is not within the invention.

Position 168	Position 206	Position 233
A/G/N/Y	A/G/N/M	A/G/N/Q

Accordingly, aspects of the invention are wherein position 206 and position 233 are both Ala (M206A Q233A) or Gly or Asn, or combinations thereof: (M206G Q233A), (M206G Q233G), (M206A Q233G), (M206N Q233A), (M206N Q233N), (M206A Q233N). Aspects of the invention are wherein postion 168 is amended according to the below:

Position 168	Position 206	Position 233
A/G/N	A	Q
A/G/N	G	Q
A/G/N	N	Q
A/G/N	M	A
A/G/N	M	G
A/G/N	M	N
A/G/N	A	A
A/G/N	G	A
A/G/N	N	A
A/G/N	A	G
A/G/N	A	N
A/G/N	N	G
A/G/N	N	N
A/G/N	G	G
A/G/N	G	N

Aspects of the invention are wherein at least one of the amended positions are amended into an Ala.

Aspects of the invention are the following mutants: (Y168G M206A), (Y168G M206A 233A) , (Y168G M206N), (Y168G M206N 233A), (Y168A M206A 233A), (Y168A M206A), (Y168A M206N), (Y168A M206N 233A) and (M206A Q233A);

The invention thus provides a novel enzymes capable of cleaving a peptide containing an initiating methionine followed by a Asn, Leu, Ile, Phe, His, Gln or Trp in the P1' postion as well as the amino acids allowed by the wild type E.coli aminopeptidase. Wildtype E.coli methionine aminopeptidase allows the P1' to be any of the following amino acids: Gly, Ala, Ser, Thr, Pro, Val or Cys. The invention thus also provides recombinant DNA molecules encoding the sequence above. The DNA sequences are disclosed in Seq. Id. no. 2, 4 and 6. The invention also provides specifically the DNA encoding the sequences for the mutants M206A, Q233A or M206A Q233A) above.

In the present invention the mutant Methionine aminopeptidases are expressed in *E. coli*, but in principle the host cells could be of other prokaryotic origin or eukaryotic origin such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris* etc. or for example mammalian cells.
The invention thus provides host cells transformed by the recombinant DNA molecule of above.
Removal of initiator methionine by methionine aminopeptidase may be performed *in vitro* following methionine aminopeptidase expression in, and purification from, prokaryotic or eukaryotic cells. This procedure is demonstrated below.
Alternatively removal of initiator methionine may take place *in vivo* either in cells expressing a di-cistronic plasmid or in cells co-expressing plasmids carrying the methionine aminopeptidase and the substrate peptide or protein. *In vivo* initiator methionine processing may also be performed in cells where the genes encoding the methionine aminopeptidase and the peptide or protein to be processed have been integrated into the genome.

Experiments have been performed which provides a set of optimum conditions for the reaction: The optimal temperature for the reaction was determined to be between 15 and 24 degrees Celsius. Typically the reaction was hereafter performed at 18 degrees Celsius.

The concentration of $ZnCl_2$ was determined to be optimal at around 7.5μM and NaCl concentration was found optimal around 100mM and acceptable under 130mM.

After cleavage of the initiator methionine separation of the product from the starting material can be achieved by exploiting the different biophysical properties of the two peptides.

In an embodiment of the invention the peptide is hIL-21, which after removal of the initial methionine contains a Gln in the N-terminal. Treatment with Qcyclase forms a pyroglutamine (pGlu). Due to cyclic amid formation the products net change is negative relative to the Methionine containing peptide. The difference in charge affects the eluation on a cation exchange column, due to Methionine containing peptide having a stronger binding to the cation resin. Further, in non-cyclised hIL-21 the N-terminal positioned Gln residue will have the ability to form a hydrogen bond between the side chain amide oxygen and the charged N-terminal backbone amine and thereby masking the charge at the N-terminus. Met-hIL-21 will not possess the ability for a similar charge masking and will therefore bind stronger to the cation exchange column than hIL-21.

In an embodiment of the invention, a method seperation of protein mixtures between identical proteins starting with Met-Gln and Gln respectively is provided.

In a specific embodiment of the invention seperation of Met-hIL-21 and hIL-21 is provided.

In another specific embodiment of the invention separation of Met-hIL-21 and hIL-21 and mutants thereof is provided.

EXAMPLES

Various Met-AP expression constructs, as outlined in figure 1, have been created. NT1 (HHHNSWDHDINR) or hexa-His tag has been added to the various mutant forms of Met-AP for purification purposes. The purification tag may be removed using Factor Xa in some constructs or Enterokinase in others, or the purification tag may be left on the enzyme. mRNA expression was under the control of the T7 or the *tac* promoter. Constructs under the control of the T7 promoter were expressed in BL21(DE3) whereas constructs under the control of the *tac* promoter were expressed in BL21. Expression was induced by addition of

IPTG to 0.4 mM to cultures (6 mL) grown to OD_{600} 0.4 in LB-medium. Cells were harvested by centrifugation after 2.5 hours. Cell lysis was done by multiple freeze-thaw cycles and soluble or insoluble protein fractions were separated by centrifugation. Soluble or insoluble protein, before or after induction of expression, originating from equal amounts cells (measured by OD_{600}) were subjected to SDS-PAGE and subsequent colloidal blue staining (Fig. 2). Met-AP expression levels were estimated at ~250 mg/L after 2.5 h of induction in 6 mL cultures.

E. coli cells harvested from 1 L of culture expressing hexa-His-Met-AP M206A, Q233A were lysed using a cell disruptor, and the clarified lysate was applied on a Ni^{2+}-NTA superflow column. Elution with an imidazole gradient released the Met-AP fusion protein at approximately 200 mM imidazole. The enzyme was further purified and buffer exchanged (into storage/cleavage buffer) using size exclusion chromatography. The enzyme was analysed using SDS-PAGE, MALDI-MS and N-terminus sequencing – verifying the molecular mass and identity of the enzyme.

According to the procedure above NT1-Enterokinase-Met-AP mutants were prepared. Expression was under the control of the tac promoter. Addition of IPTG to the cultures induced primarily soluble expression of the Met-AP enzymes. The following mutants were prepared according to the above: (Y168G M206A), (Y168G M206A 233A) , (Y168G M206N), (Y168G M206N 233A), (Y168A M206A 233A), (Y168A M206A), (Y168A M206N) and (Y168A M206N 233A)

Hexa-His-Xa-MetAP Q233A was affinity purified using Ni^{2+}-NTA superflow. Maldi-tof es mass spectrum of purified hexa-His-Xa-Met-AP M206A, Q233A shows that the correct enzymes were isolated. A mass of 32038.90 corresponds to Met-hexa-His-Xa-Met-AP M206A, Q233A and a mass of 31942.10 corresponds to hexa-His-Xa-Met-AP M206A, Q233A indicating that hexa-His-Xa-Met-AP M206A, Q233A was processed by WT Met-AP or hexa-His-Xa-Met-AP M206A, Q233A *in vivo*. The result is demonstrated in Figure 4.

Addition of hexa-His-Xa-Met-AP M206A, Q233A to Met-hIL-21 at pH 7, 18°C generated ~65 % Met-free hIL-21. In 44 h. more than 90% cleavage of Met-hIL-21 could be observed (Figure 3).

Another mutant prepared by this method was hexa-His-Xa-Met-AP Q233A;

Removal of initiator Met from Met-IL21 by Met-AP (M206A, Q233A).

Purified Met-AP (M206A, Q233A) was used to remove the initiator Methionine from partly or fully purified Met-IL21. The cleavage was performed in a reaction buffer typically consisting of the following components: 2-100 mM K_2SO_4, 2-500 mM NaCl, 1-100 µM $ZnCl_2$ and 2-30 mM Hepes buffer pH 6-8. The cleavage was assayed by MALDI-TOF spectroscopy. The time of reaction was 2-66 hours. Using these condition removal of Methionine from Met-IL21 below detection limits of Met-IL21 could be performed.

Removal of initiator Met from Met-IL21 by Met-AP (M206A).

Purified Met-AP (M206A) was used to remove the initiator Methionine from partly or fully purified Met-IL21. The cleavage was performed in a reaction buffer typically consisting of the following components: 2-100 mM K_2SO_4, 2-500 mM NaCl, 1-100 µM $ZnCl_2$ and 2-30 mM Hepes buffer pH 6-8. The cleavage was assayed by MALDI-TOF spectroscopy. The time of reaction was 2-66 hours. Using these condition removal of Methionine from Met-IL21 below detection limits of Met-IL21 could be performed.

Influence of temperature on removal of initiator Met from Met-IL21 by Met-AP (M206A, Q233A).

Using the conditions and assay described in example 1 the temperature was varied between 4, 15, 24 and 30 degrees Celsius, respectively while the other parameters was fixed. The optimal temperature for the reaction was determined to be between 15 and 24 degrees Celsius. Typically the reaction was hereafter performed at 18 degrees Celsius.

Influence of $ZnCl_2$ concentration on removal of initiator Met from Met-IL21 by Met-AP (M206A, Q233A).

Using the conditions and assay described in example 1 the $ZnCl_2$ concentration was varied between 7.5, 11 and 15 μM, respectively while the other parameters were fixed. The optimal $ZnCl_2$ concentration for the reaction was determined to be 7.5 μM. Typically, the reaction was hereafter performed at 7.5 μM $ZnCl_2$.

Influence of NaCl concentration on removal of initiator Met from Met-IL21 by Met-AP (M206A, Q233A).

Using the conditions and assay described in example 1 the NaCl concentration was varied between 80, 130 and 180 mM, respectively while the other parameters were fixed. The maximum NaCl concentration tolerated for the reaction to run was determined to be 130 mM. Typically the reaction was hereafter performed at 100 mM NaCl.

Influence of the addition of Q-cyclase on removal of initiator Met from Met-IL21 by Met-AP (M206A, Q233A) and the formation of pyro-glutamine.

Using the conditions as described in examples above the effect of adding Q-cyclase to the reaction mixture was determined. Again MALDI-TOF was used for assaying the removal of Methonine and subsequently conversion of glutamine in position 1 in IL21 into pyro-glutamine. It was found that the addition of Q-cyclase to the reaction mixture did not negatively influence the removal of ini-

tiator Methionine from Met-IL21 and further the Q-cyclase was fully efficient in converting converting glutamine in position 1 in IL21 into pyro-glutamine under the reaction conditions described in the examples above.

Purification and separation of Met-IL21, IL21 and pyro-glutamine IL-21 using a Mono-S column.

The different bio-physical properties between Met-IL21, IL21 and pyro-glutamine IL21 can be used for purification purposes/separation. Pyro-glutamine IL21 will due to the cyclized amid formation lack the normal protonation of the N-terminus. The (-1) charge difference between hIl-21 starting with pyro-glutamine and Met-IL21 can be used on a cation exchange column that will elute pyro-glutamine IL21 first (due to its lack of one positive charge) and subsequently Met-IL21 which displays a stronger binding to the cation resin. Further, in non-cyclized IL21 the N-terminally positioned glutamine will have the ability to form a hydrogen bond between the side chain amide oxygen and the charged N-terminal backbone amine, and thereby masking the charge at the N-terminus. Met-IL21 will not poses the ability for a similar charge masking and will therefore bind stronger to a cation exchange column than IL21. A mixture of Met-IL21, IL21 and pyro-glytamine IL21 including 300 mM NaCl and buffered at pH 6.5 was loaded on a Mono-S column. The A buffer consisted of 300mM NaCl buffered at pH 6.5 and the B buffer 1 M NaCl buffered at pH 6.5. A linear gradient (performed on an AKTA system) from 0-20% B buffer was applied over 45 column volumes. The fractions was assayed as described under the Q-cyclase example above. Using the above described gradient, efficient separation of Met-IL21, IL21. pyro-glutamine IL21 was achieved.(Figure 5)

Met-hGH

Purified Met-AP (M206A) is used to remove the initiator Methionine from partly or fully purified Met-hGH (human growth hormone) where the P1' is a Phe residue. The cleavage is performed in a reaction buffer typically consisting of the following components: 2-100 mM K_2SO_4, 2-500 mM NaCl, 1-100 µM $ZnCl_2$ and 2-30 mM Hepes buffer pH 6-8. The cleavage is assayed by MALDI-TOF spectros-

copy. The time of reaction is 2-66 hours. Using these conditions partly or full removal of Methionine from Met-hGH is demonstrated.

Met-hGH

Purified Met-AP (M206A, Q233A) is used to remove the initiator Methionine from partly or fully purified Met-hGH (human growth hormone) where the P1' is a Phe residue. The cleavage is performed in a reaction buffer typically consisting of the following components: 2-100 mM K_2SO_4, 2-500 mM NaCl, 1-100 µM $ZnCl_2$ and 2-30 mM Hepes buffer pH 6-8. The cleavage is assayed by MALDI-TOF spectroscopy. The time of reaction is 2-66 hours. Using these conditions partly or full removal of Methionine from Met-hGH is achieved.

Met-IL-20

Purified Met-AP (M206A) is used to remove the initiator Methionine from partly or fully purified Met-IL-20 where the P1' is a Leu residue. The cleavage is performed in a reaction buffer typically consisting of the following components: 2-100 mM K_2SO_4, 2-500 mM NaCl, 1-100 µM $ZnCl_2$ and 2-30 mM Hepes buffer pH 6-8. The cleavage is assayed by MALDI-TOF spectroscopy. The time of reaction is 2-66 hours. Using these conditions partly or full removal of Methionine from Met-IL-20 is demonstrated.

Met-IL-20

Purified Met-AP (M206A, Q233A) is used to remove the initiator Methionine from partly or fully purified Met-IL-20 where the P1' is a Leu residue. The cleavage is performed in a reaction buffer typically consisting of the following components: 2-100 mM K_2SO_4, 2–500 µM NaCl, 1–100 µM $ZnCl_2$ and 2-30 mM Hepes buffer pH 6-8. The cleavage is assayed by MALDI-TOF spectroscopy. The time of reaction is 2-66 hours. Using these conditions partly or full removal of Methionine from Met-IL-20 is demonstrated.

CLAIMS

1. *E. coli* aminopeptidease variants which are mutated in the substrate defining pockets.

2. *E. coli* aminopeptidase variants which are mutated in the active site having extended substrate specificity the P1' position relative to the wild type.

3. The E.coli methionine aminopeptidase variants according to any of the claims 1-2 which extend the substrate specificity in P1' position to include Asn, Leu, Ile, Phe, His, Gln or Trp as well as the aminoacids allowed in position P1' by the wild type.

4. E.coli methionine aminopeptidases of any of the claims 1-3 wherein the residues in positions 168, 206 or 233 has been amended into a sequence different from Y168 and M206 and Q233.

5. E.coli methionine aminopeptidases according to any of the claims 1-4, comprising amendments of the amino acid in position 168.

6. E.coli methionine aminopeptidases according to any of the claims 1-4, comprising amendment in position 206.

7. E.coli methionine aminopeptidases according to any of the claims 1-4, comprising amendment in position 233.

8. E.coli methionine aminopeptidases according to any of the claims 1-4 and 6-7, comprising amendments in position 206 and 233.

9. E.coli methionine aminopeptidases according to any of the claims 1-6, comprising amendments in position 168 and 206.

10. E.coli methionine aminopeptidases according to any of the claims 1-5 and 7, comprising amendments in position 168 and 233.

11. E.coli methionine aminopeptidase according to any of the claims 1-10 comprising amendments in positions 168, 206 and 233.

12. E.coli methionine aminopeptidases according to any of the claims 1-11 wherein the amendments comprises exchange of wildtype amino acid into Gly, Ala, Ser, Thr, Asn or Asp.

13. E.coli methionine aminopeptidases according to claim 12, wherein the amendments comprises Ala and/or Gly.

14. E.coli methionine aminopeptidases according to claim 13 wherein the amendments comprises Ala.

15. E.coli methionine aminopeptidase according to any of the claims 5-14 wherein position 168 is Ala.

16. E.coli methionine aminopeptidase according to any of the claims 5-14 wherein position 206 is Ala.

17. E.coli methionine aminopeptidase according to any of the claims 5-14 wherein position 233 is Ala.

18. E.coli methionine aminopeptidase according to any of the claims 5-14 wherein position 206 and 233 are both Ala.

19. An E.coli methionine aminopeptidase having the amino acid sequence shown in Seq. id. No. 3;

20. An E.coli methionine aminopeptidase having the amino acid sequence shown in Seq. id. No. 5;

21. An E.coli methionine aminopeptidase having the amino acid sequence shown in Seq. id. No. 7;

22. An E.coli methionine aminopeptidase having the amino acid sequence shown in Seq. id. No. 9;

23. The use of an aminopeptidase of any of the claims 1-22 for removal of initiator methionine from polypeptides.

24. The use according to claim 23, wherein the peptide is IL-21, mutants, chemical derivatives or species homologues thereof; or IL-20 or hGH.

25. The use according to claim 24, wherein the peptide is human IL-21.

26. The use according to claim 24, wherein the peptide is mutants of human IL-21.

27. The use according to claim 24, wherein the peptide is chemical derivatives of human IL-21.

28. The use according to claim 24, wherein the peptide is species homologues of human IL-21.

29. Isolated DNA encoding the peptides of claims 1-22.

30. The isolated DNA encoding the peptide of claim 19 as shown in Seq. id. no. 2

31. The isolated DNA encoding the peptide of claim 20 as shown in Seq. id. no. 4

32. The isolated DNA encoding the peptide of claim 21 as shown in Seq. id. no. 6

33. The isolated DNA encoding the peptide of claim 21 as shown in Seq. id. no. 8

34. A host cell comprising any of the nucleotide sequences of claims 29-33.

35. A host cell according to claim 34, comprising also the nucleotide sequences encoding the Met-containing peptide.

36. The host cell according to claim 35 wherein the sequences are in the same plasmid.

37. The host cell according to claim 35 wherein the sequences are on different plasmids.

38. A method for purification of the product peptide having Gln in the N-terminus from the substrate, the Met-Gln containing peptide, comprising the step of applying Qcyclase followed by purification utilising the charge difference of the compounds.

39. The method of claim 38 comprising purification on a cation exchange column.

Fig. 1

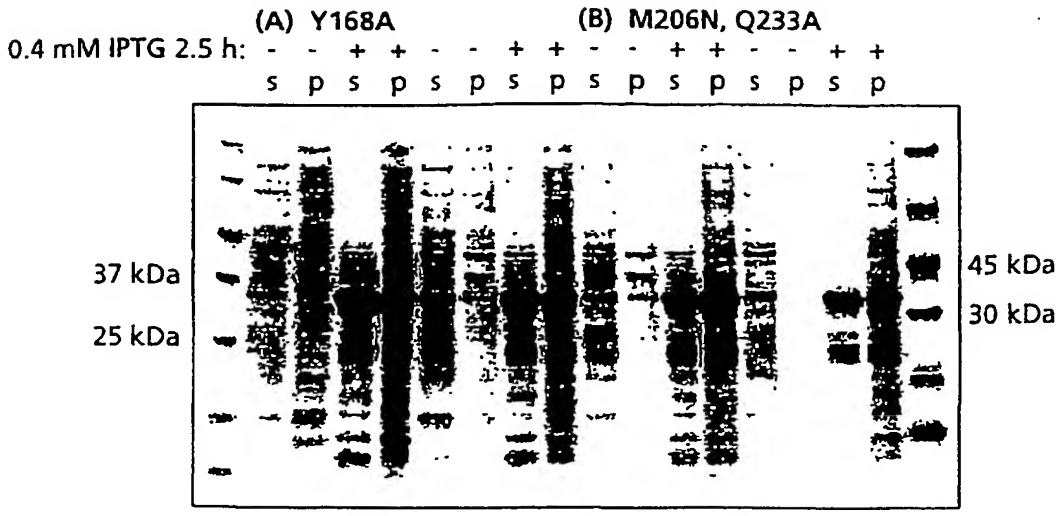
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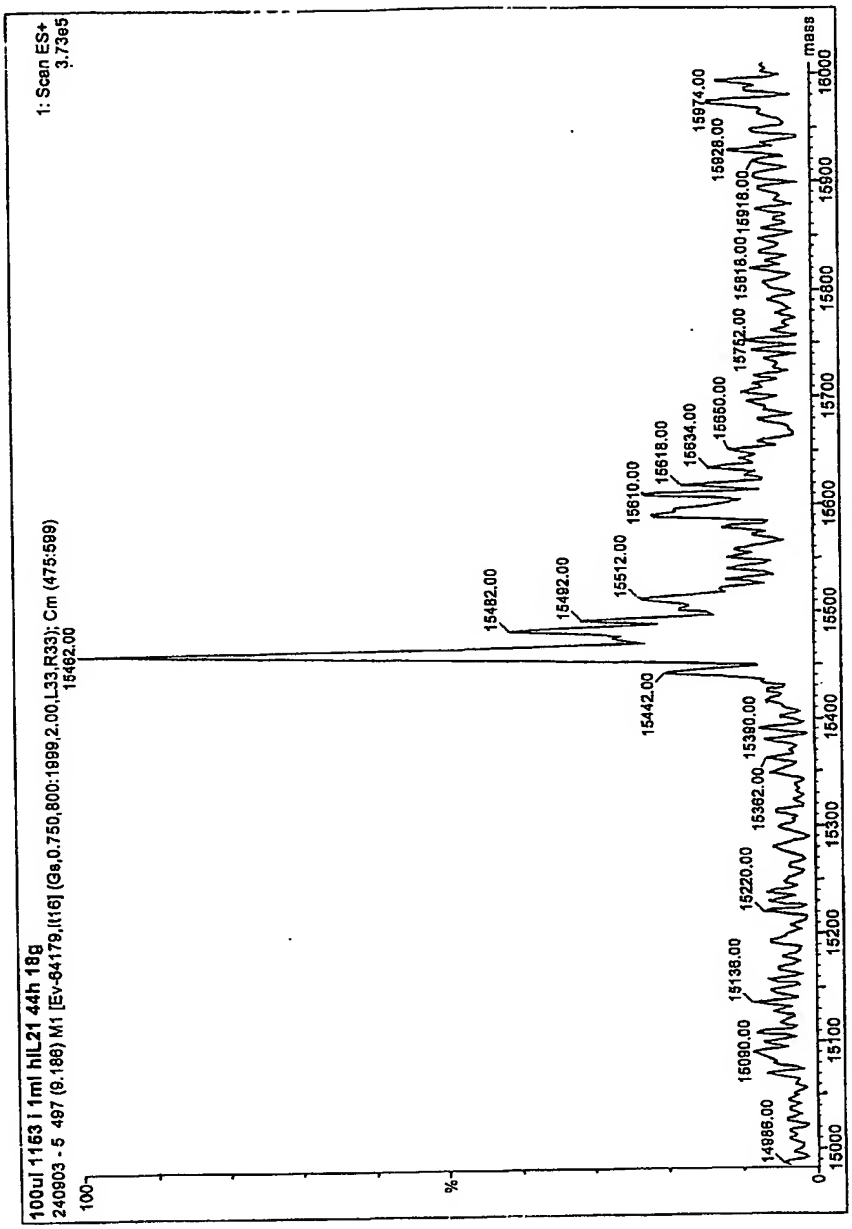
Fig. 2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240903 - 5 497 (9.188) M1 [Ev-84179,I(16] (Gs,0.750,800:1899,2.00,L33,R33); Cm (475:599)
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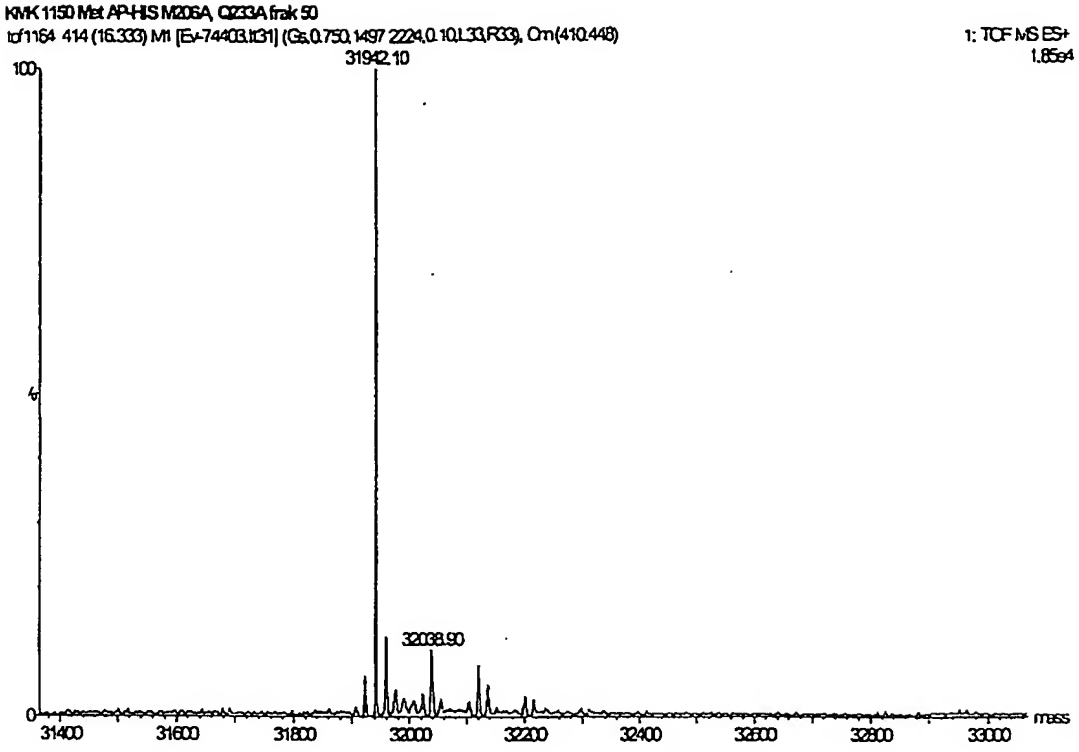
%
mass

Fig. 3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1: TOF MS ES+
1.85e4
31942.10
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mass

Fig. 4

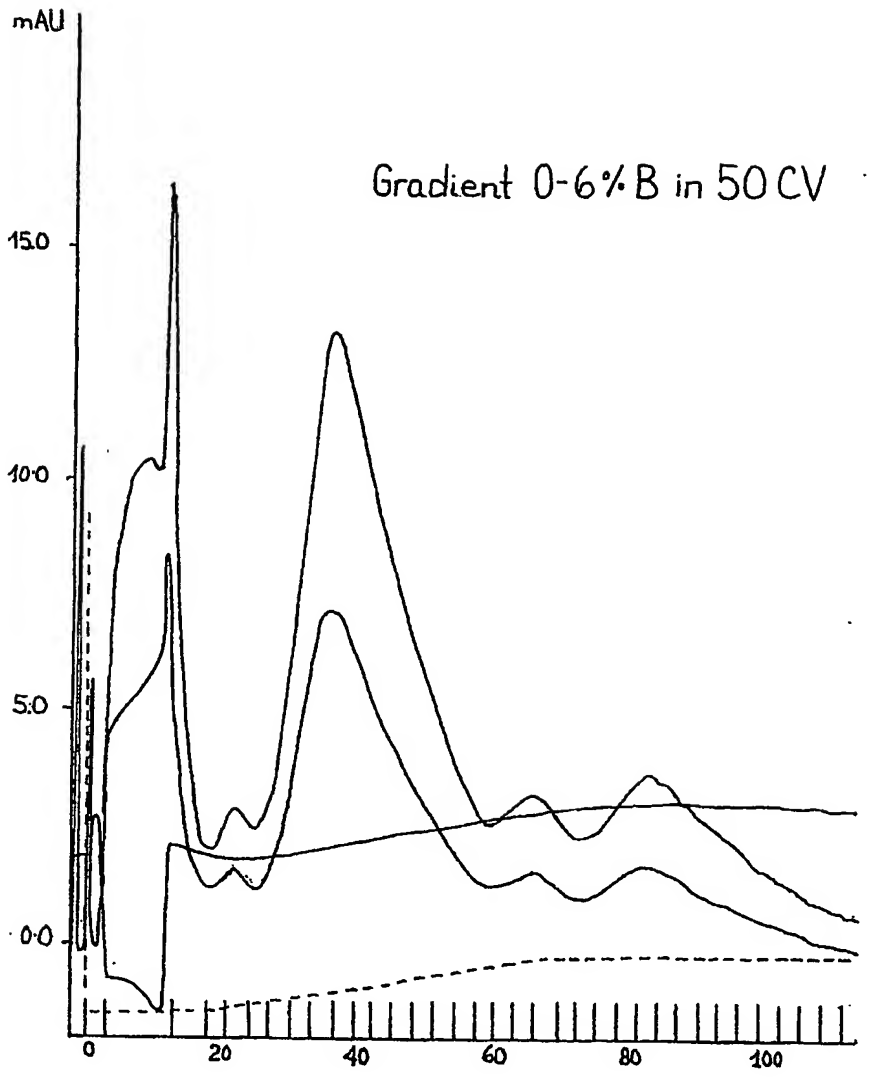

Fig. 5

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Tyr Pro Lys Ser Val Cys Ile Ser Ile Asn Glu Val Val Cys His Gly
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2004/000891

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBASE, FSTA, BIOSIS, Sequence Search, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LOWTHER W T ET AL: "Structure and function of the methionine aminopeptidases" BIOCHIMICA ET BIOPHYSICA ACTA. PROTEIN STRUCTURE AND MOLECULAR ENZYMOLOGY, ELSEVIER, AMSTERDAM,, NL, vol. 1477, no. 1-2, 7 March 2000 (2000-03-07), pages 157-167, XP004278892 ISSN: 0167-4838 the whole document	1-37

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 March 2005	23/03/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Piret, B

Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHIU CHEN-HSIANG ET AL: "Amino acid residues involved in the functional integrity of Escherichia coli methionine aminopeptidase" JOURNAL OF BACTERIOLOGY, vol. 181, no. 15, August 1999 (1999-08), pages 4686-4689, XP002320613 ISSN: 0021-9193 the whole document	1,12
X	WALKER K W ET AL: "Yeast methionine aminopeptidase I. Alteration of substrate specificity by site-directed mutagenesis." THE JOURNAL OF BIOLOGICAL CHEMISTRY. 7 MAY 1999, vol. 274, no. 19, 7 May 1999 (1999-05-07), pages 13403-13409, XP002320532 ISSN: 0021-9258 the whole document & WALKER K W ET AL: "Erratum: Yeast methionine aminopeptidase I. Alteration of substrate specificity by site-directed mutagenesis (Journal of Biological Chemistry (1999) 274 (13403-13409))" JOURNAL OF BIOLOGICAL CHEMISTRY 17 SEP 1999 UNITED STATES, vol. 274, no. 38, 17 September 1999 (1999-09-17), page 27338, ISSN: 0021-9258	1-37
A	LOWTHER W T ET AL: "Escherichia coli methionine aminopeptidase: implications of crystallographic analyses of the native, mutant, and inhibited enzymes for the mechanism of catalysis." BIOCHEMISTRY. 15 JUN 1999, vol. 38, no. 24, 15 June 1999 (1999-06-15), pages 7678-7688, XP002320531 ISSN: 0006-2960 the whole document	1-37
P,X	LIAO Y-D ET AL: "REMOVAL OF N-TERMINAL METHIONINE FROM RECOMBINANT PROTEINS BY ENGINEERED E. COLI METHIONINE AMINOPEPTIDASE" PROTEIN SCIENCE, CAMBRIDGE UNIVERSITY PRESS, CAMBRIDGE, GB, vol. 13, no. 7, July 2004 (2004-07), pages 1802-1810, XP008043171 ISSN: 0961-8368 the whole document	1-37

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2004/000891

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	LIU L-F ET AL: "Escherichia coli methionine aminopeptidase with Tyr168 to alanine substitution can improve the N-terminal processing of recombinant proteins with valine at the penultimate position" ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS, NEW YORK, NY, US, vol. 329, no. 2, 15 June 2004 (2004-06-15), pages 345-347, XP004509836 ISSN: 0003-2697 the whole document	1-37
A	WO 99/61617 A (HUMAN GENOME SCIENCES, INC; RUBEN, STEVEN, M; EBNER, REINHARD) 2 December 1999 (1999-12-02)	23-28

INTERNATIONAL SEARCH REPORT

Information on patent family members

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